

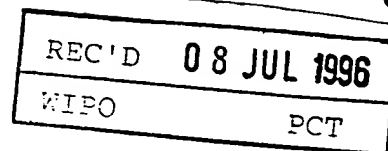
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Avgift
Fee

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ULTRASENSITIVE IMMUNOASSAYS

The present invention relates to ultrasensitive immunoassays. More specifically, it relates to immunological test kits and processes for immunological detection of a specific antigen. In the present invention, the fields of immunology and molecular genetics, respectively, are combined.

Immunoassays represent powerful tools to identify a very wide range of compounds, such as antigens and antibodies. Examples of immunoassays are ELISA (enzyme linked immuno assay), EIA (enzyme immunoassay), and RIA (radio immunoassay). Common to all these immunoassay, is that detection sensitivity is limited by the affinity of typical antibodies.

With the prior art immunoassays, detection is not possible below a certain number of molecules, because the background, i.e. unspecifically bound material, interferes with the results. Detection of very low numbers of antigen is becoming increasingly important, especially for diagnostic applications. Therefore, further developments in sensitivity as well as specificity of immunological assays are desired.

Cantor et al, Science, Vol., 258, 2 Oct. 1992, have previously reported the attachment of oligonucleotides to antibodies in order to permit detection of such antibodies having bound antigen in immune reactions. A streptavidin-protein A chimera that possesses tight and specific binding affinity for both biotin and immunoglobulin G was used to attach biotinylated DNA specifically to antigen-monoclonal antibody complexes that had been immobilized on microtiter plate wells. Then, a segment of the attached DNA was amplified by PCR. Analysis of the PCR products by agarose gel electrophoresis after staining with ethidium bromide Allowed detection of 580 antigen molecules (9.6×10^{-14} moles) which is a significant improvement compared to, for example, conventional ELISA.

However, in Cantor et al., the labeled DNA-antibody complexes are assembled in situ during the assay. This can create variable stoichiometry in the assembly of the components and in the attachment of the DNA label. Moreover, extra steps are required for addition of biotinylated reagents and binding proteins. Numerous wash steps are also needed to remove excess reagents and to free assay components of non-specifically bound reagents.

Hendrickson et al., Nucleic Acids Research, 1995, Vol 23, No.3, report an advancement of the Cantor et al. assay that reduces complexity. This is achieved through labeling antibody with DNA by direct covalent linkage of the DNA to the antibody. In this approach, the analyte specific antibody and the 5' amino modified DNA oligonucleotide are independently activated by means of separate heterobifunctional cross-linking agents. The activated antibody and DNA label are then coupled in a single spontaneous reaction.

International patent publication no. WO 91/17442 describes a molecular probe for use as a signal amplifier in immunoassays for detecting i.a. antigens. The probe comprises an antibody, a double stranded polynucleotide functioning as a promoter for a DNA dependend RNA polymerase, and a single or double stranded template for the promoter. The transcription product is quantified and correlated to the amount of present antigen in a sample.

However, in all three of the above described immunoassays the attached DNA is only used as a marker by being amplified to detectable levels. There is no distinction between oligonucleotides attached to antibodies having bound antigen and oligonucleotides attached to antibodies not having bound antigen, i. . those being non-specifically trapped. Non-specifically trapped antibodies give rise to an undesired background signal and limits the minimum number of antigen molecules that can be detected and it will not be possible to distinguish between false positive and true pos.civ results below a certain number of antigen molecules. Commonly, solid supports such as microtiter plates, are used for the reactions. According to prior art, there will always be an excess of oligonucleotide-labeled antibody that cannot be

removed from the solid support by adding background-lowering agents and by repeated wash steps.

According to a first aspect of the invention, there is provided an immunological test kit comprising a first immobilized reagent having affinity to a specific macromolecule, such as a protein. Furthermore, the test kit comprises a second and a third affinity reagent specific for different determinantes of said protein, and modified with crosslinkable compounds enabling a) conjugation of said second and third affinity reagent only when both are bound to the said, same macromolecule, and b) detection by amplification.

According to a preferred embodiment of the invention, the affinity reagents are antibodies and the crosslinkable compounds are oligonucleotide extensions attached to the second and third antibody, ^{respectively}. The macromolecule is in this case a specific antigen.

According to a second aspect of the invention there is provided an immunoassay for detection of a specific antigen, comprising the following steps:

- adding a sample suspected of containing said specific antigen to a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen,
- incubating,
- removing unreacted sample,
- adding a second and a third antibody specific for a second and third epitope of said antigen, and modified with crosslinkable compounds enabling conjugation of said second and third antibody when both are bound to the said, same antigen,
- incubating,
- removing excess of second and third antibody,
- amplifying said crosslinked compounds, and
- detecting the amplified products.

Products from the amplification reaction only result when two antibodies, i.e. the second and the third, have bound to the same antigen. Thus, amplification is specific for antibodies having bound to antigen. Non-specifically trapped antibodies do not give

rise to any signal.

Extremely low numbers of antigenic molecules can be detectable using the immunoassay according to the invention, down to a single molecule. Thus, the present invention enable reliable immunoassays in situations where insufficient numbers of antigens are available for conventional assays.

The present invention will be described more detailed below with reference to the accompanying drawings, in which

Fig. 1 is a schematic view of the principles of the immunoassay according to the invention, and

Fig. 2 shows chemical coupling of amino-modified oligonucleotides to macromolecules.

In Fig. 1 there is shown an immobilized antibody to a specific antigen applied together with two other antibodies, specific for other determinants on the same antigen. Besides antibodies other specifically interacting species with a known affinity, such as lectins, receptors, single chain antibodies, cofactors, oligonucleotides and other non-proteins, can be used in the invention.

The interacting species are modified with crosslinkable compounds in the form of an interacting pair, preferably short oligonucleotide extensions. Upon the coordinated binding of several so modified antibodies, oligonucleotides of neighbouring antibodies are conjugated to each other. The conjugation may or may not necessitate an enzymatic ligation step depending on the orientation of the oligonucleotide extensions.

If the conjugation is between free 3' and 5' ends ligation is necessary, such as by T4 RNA ligase or T4 DNA ligase. To facilitate the conjugation, it is convenient to use a stretch of oligonucleotides base pairing to and, thereby, juxtaposing the free ends of the oligonucleotides and permitting their joining

If the conjugation is between free 3' ends these have to be designed to be mutually complementary to achieve base pairing and initiation of DNA synthesis extending the 3' ends of the the molecules.

Thus, only in those cases where the antibodies are brought close enough through binding to the same antigen molecule the oligonucleotides can be ligated. Ligated molecules subsequently serve as templates for nucleic acid amplification reactions.

In Fig. 2, there is shown a suitable way to attach the oligonucleotide extension to the antibodies. First, the oligonucleotides are terminally amino-modified and then attached to primary amines on the antibodies via disulphide bonds, e.g. according to the technique of Chue and Orgel, Nucleic Acid Research, Vol. 16, No. 9, 1988. Another way is by direct covalent coupling as described by Hendrickson et al., supra.

The antibodies used in the invention can be polyclonal, monoclonal or single chain antibodies produced by bacteriophages. In the latter case, it is possible to have antibodies equipped with an oligonucleotide binding part, rendering the above coupling step between antibody and oligonucleotide unnecessary.

The amplification technique to obtain detectable products are selected from PCR (polymerase chain reaction), LCR (ligase chain reaction), SDA (Strand Displacement Amplification) bacteriophage ϕ replication, and 3SR (Self-Sustained Synthetic Reaction), of which the latter three methods do not require temperature cycling.

The method for detecting amplified products can, for example, be direct incorporation of a label, such as radioisotopes, fluorochromes, and enzymes, into the amplified products with the use of label-conjugated primers or nucleotides. Preferably, the accumulation of amplified products is monitored via the fluorescence from intercalating dyes, such as Cybergreen.

The invention is not restricted to any special kind of macro-

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molecule, such as an antigen; the only criterion it has to fulfill is that it must be able to bind three antibodies/affinity reagents. In the case where the affinity reagents are antibodies, the three antibodies are specific for different epitopes on the antigen. By biosensor analysis, it is possible to assure that the antibodies do not bind to overlapping epitopes on the antigen.

Examples of macromolecules are human myoglobin and human growth hormone. Ultrasensitive assays for growth hormone will have significant value in clinical situations where hormone levels are undetectable by prior art assays.

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CLAIMS

1. An immunological test kit comprising a first immobilized reagent having affinity to a specific macromolecule, characterized in a second and a third affinity reagent specific for different determinantes of said macromolecule, and modified with crosslinkable compounds for a) conjugation of said second and third affinity reagent when both are bound to the said, same macromolecule, and b) detection by amplification.
2. An immunological test kit according to claim 1, characterized in that the crosslinkable compounds are oligonucleotides.
3. An immunological test kit according to claim 1, characterized in that the affinity reagents are antibodies, and that the macromolecule is a specific antigen.
4. An immunological test kit according to claims 1 or 2, characterized in that the affinity reagents are lectins, receptors, single chain antibodies, cofactors and nucleic acids.
5. An immunological test kit according to any of the claims 1-4, characterized in that it further comprises a ligase.
6. An immunoassay for detection of a specific antigen, characterized in:
 - adding a sample suspected of containing said specific antigen to a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen,
 - incubating,
 - removing unreacted sample,
 - adding a second and a third antibody specific for a second and third epitope of said antigen, and modified with crosslinkable compounds enabling conjugation of said second and third antibody when both are bound to the said, same antigen,
 - incubating,
 - removing excess of second and third antibody,
 - amplifying said crosslinked compounds, and
 - detecting the amplified products

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ABSTRACT

The present invention relates to immunological test kits and processes for immunological detection of a specific antigen. By combining immunology and molecular genetics, the invention provides extremely sensitive immunoassays. There is substantially no background and detection is possible down to single molecules.

(Fig. 1)

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FIG. 1

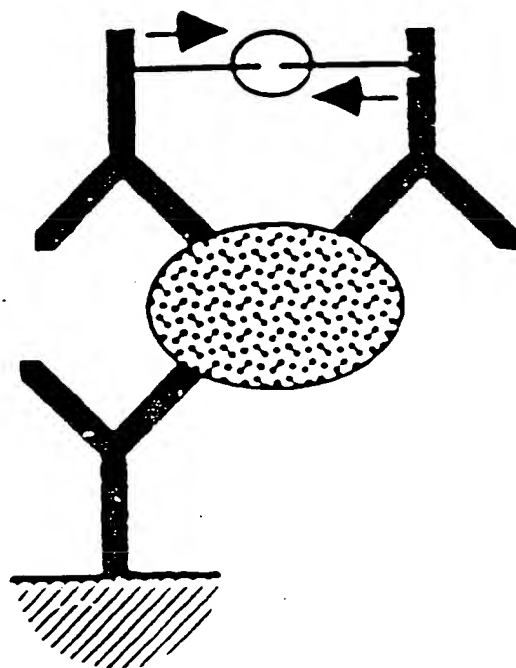


FIG. 2

